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Oxidative Activity of Human Polymorphonuclear Leukocytes Stimulated by the Long-Chain Phosphatidic Acids

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Summary

It has already been suggested that phosphatidic acids (PAs) play an important role in the regulation of signaling pathways involved in the production of reactive oxygen species (ROS) by human polymorphonuclear leukocytes (PMNs). The present study was performed to elucidate the effects of extracellularly added PAs – 1,2-distearoyl- (DSPA) and 1-stearoyl-2-arachidonoyl-*sn*-glycero-phosphate (SAPA) – on the ROS production and on the elastase release by human PMNs. ROS production was monitored by luminol-amplified chemiluminescence and the elastase activity was measured in the supernatant of the PA-stimulated human PMNs by colorimetric assay. Obtained effects were compared with those of cells stimulated by either a chemotactic tripeptide, phorbol ester or calcium ionophore. Our results show that long-chain PAs at concentrations higher than 3×10^{-5} mol/l stimulate the ROS production by human PMNs, whereas they were ineffective in promoting the elastase release. The chemiluminescence pattern of the SAPA-stimulated cells exhibited a biphasic curve, whereas cell stimulation with DSPA resulted in a monophasic chemiluminescence curve. Stimulation of the ROS production by PAs in dependence of the fatty acid composition required the activity of protein kinases.

Key words

Elastase release • Human PMNs • Luminol-amplified chemiluminescence • Phosphatidic acid • Signal transduction

Introduction

Reactive oxygen species (ROS) and enzymes released from stimulated polymorphonuclear leukocytes (PMNs) at the site of infection are involved in the defence against invading microorganisms (Fantone and Ward 1982). On the other hand, they also may contribute to tissue damage in some diseases (Prasad *et al.* 1990, Nurcombe *et al.* 1991, Schiller *et al.* 1996, 2000).

Several phospholipases that are activated in stimulated PMNs generate important lipid second messengers involved in the control of PMN functions (Watson 1996). Diacylglycerols (DAGs) that are produced in the first steps directly activate protein kinase C (PKC). It is generally accepted that during the respiratory burst of PMN, a continuous supply of the cells with DAGs is provided and that all DAG species are able to activate PKC and subsequently the NADPH oxidase as the first step in the ROS production. Data from our group have indicated that the above-mentioned hypothesis is questionable, as DAGs with saturated fatty acids cannot stimulate the respiratory burst response (Arnhold *et al.* 1999). Another lipid derived second messenger, phosphatidic acid (PA) also plays an important role in the

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regulation of the PMN activity. PAs activate serine/threonine protein kinase (phosphatidic acid-activated protein kinase, PAPK) that also phosphorylates and activates several components of NADPH oxidase (Waite *et al.* 1997).

PAs are also assumed to play an important role in intercellular communication and they are important inflammatory mediators (English 1996). It has been shown that PAs released from activated platelets increase endothelial cell migration and platelet aggregation (Xiao *et al.* 2001). Finally, PA and the corresponding lyso-compound, LPA, were shown to represent chemotactic factors for monocytes and PMNs (Zhou *et al.* 1995). It has been shown that the short-chain PAs are recognized by a specific receptor on the PMN plasma membrane, the specificity of which decreases with increasing chain length of the fatty acid residues (Siddiqui and English 1996). PA action requires intracellular PA production by the activity of phospholipase D (PLD), as well as tyrosine kinase and phosphatidylinositol 3-kinase (PI 3-K) activation, and the opening of calcium channels (Siddiqui and English 1996, 1997, 2000, Siddiqui *et al.* 2000). PA is also implicated to play a role in regulating a number of intracellular processes by interacting with different enzymes, e.g. phospholipase C (PLC), PLD or Raf-1 (Geng *et al.* 1998, English 1996, Jones *et al.* 1993).

Our previous studies by luminol-amplified chemiluminescence (CL) have shown that PKC activation is required for the initial phase of the respiratory burst response of human PMNs stimulated by a chemotactic factor, whereas the later phase was influenced to a higher extent by the PAs (Arnhold *et al.* 1999). In the present work, we have studied the effects of exogenous long-chain PAs on the respiratory burst activity as well as the PMN degranulation. Our results indicate that exogenously added PAs stimulate the oxidative activity of PMNs, but are unable to induce the degranulation of azurophilic granules.

Methods

Chemicals

Chemicals for PMN purification, i.e. dextran, Ficoll-hypaque solution and Hank's balanced salt solution (HBSS) and the stimulators, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), phorbol myristate acetate (PMA) and calcium ionophores – A23187 and ionomycin – were from Sigma-Aldrich GmbH (Deisenhofen, Germany). Heparin from porcine mucosa was from Fluka (Seelze, Germany). Luminol (5-amino-2, 3-dihydrophthalazine-1,4-dione) was a product of Boehringer-Mannheim (Mannheim, Germany). Inhibitors used in CL experiments: bisindolylmaleimide I and staurosporine were from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Stock solutions of those inhibitors were made in dimethylsulfoxide (DMSO). NaF was purchased from Sigma-Aldrich GmbH (Deisenhofen, Germany) and was dissolved in HBSS. The substrate for the determination of elastase activity, N-methoxy-succinyl-Ala-Ala-Pro-Val-p-nitroanilide (p-nitroanilide substrate) was from Sigma-Aldrich GmbH (Deisenhofen, Germany). Phospholipids (PLs) used in this study: 1,2-distearoyl-sn-glycero-3-phosphate, (DSPA), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphate (SAPA) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were obtained as 10 mg/ml chloroform solutions from Avanti Polar Lipids Inc. (Alabama, USA). All solvents (DMSO, chloroform and methanol), 2,5-dihydroxybenzoic acid (DHB) and trifluoroacetic acid (TFA) were purchased from Fluka (Seelze, Germany) of the highest commercially available purity and were used without further purification.

Cell preparation

PMNs were isolated from heparinized (10 U/ml) blood of healthy volunteers as described earlier (Bøyum 1964). This included the dextran-enhanced sedimentation of erythrocytes, lysis of remaining red blood cells, and the Ficoll-density gradient centrifugation. PMNs obtained in the pellet were resuspended in HBSS and stored on ice until use. PMNs were used within 2 h after purification. The cell viability was tested by the Trypan blue exclusion test.

Preparation of phospholipids

Chloroform was removed from the PA or PC solutions by vacuum evaporation (Vacuum Evaporator, Jouan, Germany) and PLs (2 mg/ml) resuspended in 10 mmol/l tris-hydroxymethyl-aminomethane (TRIS) containing 100 mmol/l NaCl (pH 9.1) by sonication (Branson SONIFIER, Germany). PL suspensions were stored at 4 °C and were used diluted with HBSS within three days after preparation. PLs were diluted in HBSS prior to application to the neutrophil suspension.

Luminol-amplified chemiluminescence of human PMNs

All chemiluminescence (CL) measurements were performed on a microplate luminometer

MicroLumat LB 96 P (EG & G Berthold, Wildbad, Germany). Freshly prepared human PMNs (10^5 cells /well) were preincubated with 5×10^{-5} mol/l luminol for 2 min at 37 °C prior to stimulation either with various concentrations of PA or with the following stimulators: fMLP (10^{-6} mol/l), PMA (10^{-7} mol/l) or ionomycin (10^{-5} mol/l). PAs were added manually into the PMN suspension to avoid their loss in the injector tube, whereas other stimulators were injected. For some experiments, PMNs were additionally incubated with inhibitors: bisindolylmaleimide I, an inhibitor of PKC (Toullec *et al.* 1991) was applied at a concentration of 1×10^{-6} mol/l, staurosporine, an inhibitor of serine/threonine protein kinases (Nishimura and Simpson 1994) was used at 0.1×10^{-6} mol/l concentration, finally, NaF an inhibitor of ecto-phosphatidate phosphohydrolase (PPH) (Taylor *et al.* 1993) was tested at two concentrations, 1×10^{-2} mol/l and 2×10^{-2} mol/l.

Measurement of the elastase activity

The elastase release test was performed according to previously described methods (Bieth *et al.* 1974, Castillo *et al.* 1979). Shortly, PMNs were suspended in HBSS at a concentration of 5×10^6 cells/ml and were stimulated by either PAs (6×10^{-5} mol/l) or with the following stimulators: fMLP (10^{-6} mol/l), a calcium ionophores, A23187 and ionomycin (10^{-5} mol/l both) or with PMA (10^{-7} mol/l). After 15 min at 37 °C the reaction was terminated by the addition of ice-cold HBSS and the cells were immediately spun down by a short centrifugation at 800 rpm for 5 min at 4 °C. Elastase activity in the supernatants was measured by the addition of 200 µl of 0.5 mmol/l *p*-nitroanilide substrate (N-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide) buffered in 0.1 mol/l HEPES / 0.5 mol/l NaCl (pH 7.4) to 50 µl of the cell supernatant. Absorbance of p-nitroanilide was measured at 405 nm and results were expressed as specific activity per 10^6 cells (U / 10^6 cells).

To test whether PAs influence the elastase activity, the human PMNs were stimulated with the calcium ionophore, A23187 (10^{-5} mol/l) for 15 min. After the reaction was terminated and the cells were spun down, various concentrations of PAs were added to the supernatant and the elastase activity was compared with the control, i.e the activity in the supernatant of the A23187-stimulated PMNs without the addition of PAs.

Extraction of lipids from human PMNs

Freshly prepared human PMNs (10^7 cells/ml) were pre-warmed at 37 °C for 5 min. After that DSPA or SAPA (6×10^{-5} mol/l in HBSS) or buffer used for the PL preparation (TRIS / HBSS) was added to the suspension. The incubation time of the PMNs with PAs was 5 or 15 min at 37 °C. Afterwards the cells were spun down by a short centrifugation and cellular pellet washed three times with 10 ml of HBSS. The Bligh and Dyer (1959) extraction procedure was applied for the lipid extraction. Briefly, a chloroform/methanol/ mixture was added to the PMN suspension in distilled water (chloroform: methanol: water with PMNs = 1:2:0.9, volume ratios) and the samples were vigorously vortexed. The chloroform layer obtained by a centifugation was used for matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.

Mass Spectrometry

All MALDI-TOF measurements were performed with 2,5-dihydroxybenzoic acid (DHB) as a matrix under conditions described elsewhere (Schiller *et al.* 1999). The residual lipids after chloroform removal from the organic extract of PMNs were resuspended in the matrix solution. After that, a 1.8 µl of the sample was applied onto the sample plate and was immediately dried under a moderate warm stream of air. All MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry workstation (PerSeptive Biosystems, Framingham, MA, USA). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. Other conditions for MALDI-TOF MS can be found elsewhere (Schiller *et al.* 1999).

Results

Luminol-amplified chemiluminescence

Figure 1a shows typical CL properties of human PMNs stimulated with fMLP, PMA or ionomycin. Reponse of PMNs to fMLP is a biphasic curve, whereas response to the both PMA and ionomycin is monophasic. The first CL phase starts immediately after the stimulation with fMLP (this phase is emphasized by a grey bar in the Fig. 1a) and lasts for about 5 min. The second phase is of somewhat lower intensity than the first one, but lasts longer. In contrary to the stimulation with fMLP, in the CL curves obtained after stimulation with PMA and/or ionomycin, it is possible to distinguish only one phase (one peak).

CL properties of human PMNs stimulated with DSPA or with SAPA are given in Figures 1b and 1c, respectively. According to its shape the CL response of the cells to PA with two saturated fatty acid residues

corresponds to the curve obtained the cell stimulation with PMA or ionomycin, but with approx. 10 times lower intensity. A biphasic curve was obtained after PMN stimulation with SAPA (Fig. 1c).

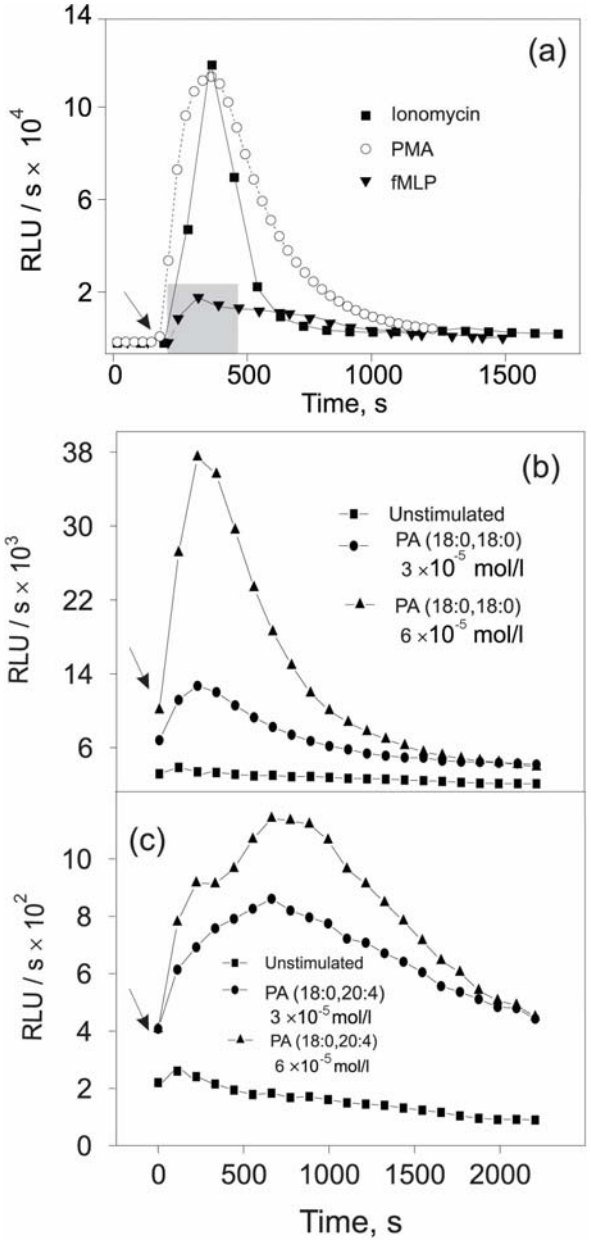

Fig. 1. CL response of PMNs stimulated with fMLP, PMA and ionomycin (**a**), DSPA (**b**) and SAPA (**c**). First CL phase in the fMLP-induced response is emphasized by a grey bar. Freshly prepared human PMNs (10^5 cells/well) were incubated with luminol (5×10^{-5} mol/l for 5 min at 37 °C. Stimulators or PAs were added to the cell suspension at the time point indicated by an arrow and the CL was monitored during 25 min after stimulation. The results represent typical data of three independent cell preparations.

The addition of pure buffer as well as of PC vesicles to the PMN suspension did not induce any CL response nor influence the CL pattern of the fMLP-stimulated PMNs (data not shown).

Figure 2 shows the CL curves obtained upon the stimulation of PMNs with DSPA (a) and SAPA (b) in the presence of bisindolylmaleimide I, a selective inhibitor of protein kinase C (PKC) (Toullec *et al.* 1991) and staurosporine, an inhibitor of serine/threonine protein kinases (Taylor *et al.* 1993). Bisindolylmaleimide I (10^{-6} mol/l) significantly inhibited the respiratory burst response of PMNs only if PMNs were stimulated by SAPA (Fig. 2b), and to a much lesser extent if PMNs were stimulated by DSPA (Fig. 2a). Staurosporine (0.2×10^{-6} mol/l) abolished the respiratory burst response completely irrespectively which PA was used for PMN stimulation (Fig. 2).

Figure 2c shows the CL response of PMNs stimulated with DSPA and the CL of the PMNs stimulated with SAPA is presented in Figure 2d. In both cases PMNs were treated with 10^{-2} mol/l NaF prior to stimulation. Obviously, the effect of inhibition with NaF is by far more pronounced when the cells were stimulated with SAPA – particularly in the first CL phase – than with DSPA.

Elastase release

The elastase activity in the supernatant of DSPA and SAPA stimulated PMNs given in Fig. 3a. The elastase activity was reduced to about 80 % of the activity measured in the supernatant of unstimulated cells. Control measurements did not reveal any reduction of elastase activity when both PAs were directly added to the supernatant of A23187-stimulated cells (data not shown).

The elastase activity in supernatant of fMLP-, A23187- and PMA-stimulated PMNs is shown in Fig. 3b. It is obvious that the elastase activity was at least four orders of magnitude higher than that measured in the supernatant of the PA-stimulated cells (Fig. 3a). The highest elastase activity was obtained in the supernatant of A23187-stimulated PMNs.

MALDI-TOF mass spectrometry of the organic extracts of PMNs

To investigate the lipid composition of PMNs after incubation with PAs and to detect DAGs and/or other metabolites possibly generated from the applied PAs, we also analyzed the organic extracts of PMNs by MALDI-TOF MS. PAs and DAGs can be easily differentiated since they yield a characteristic spectrum in

the positive ion detection mode. DAGs always exhibit only one peak corresponding to the Na^+ adduct, or one additional peak in the presence of higher K^+ concentration. The proton adducts are present with extremely low intensity (Benard *et al.* 1999). However, PAs as double negatively charged lipids require compensation with two positive ions, and cationisation with one additional ion to be detectable as singly charged positive ions (Petković *et al.* 2001a). Strongly depending on the cation concentration in the solvents used for mass spectrometry various peaks are expected in the positive ion mass spectra (Petković *et al.* 2001b). Therefore, we were looking for at least two PA peaks – in most cases the ($2\ H^+ + Na^+$) or ($H^+ + 2\ Na^+$)-adducts – in order to make the peak assignment certain.

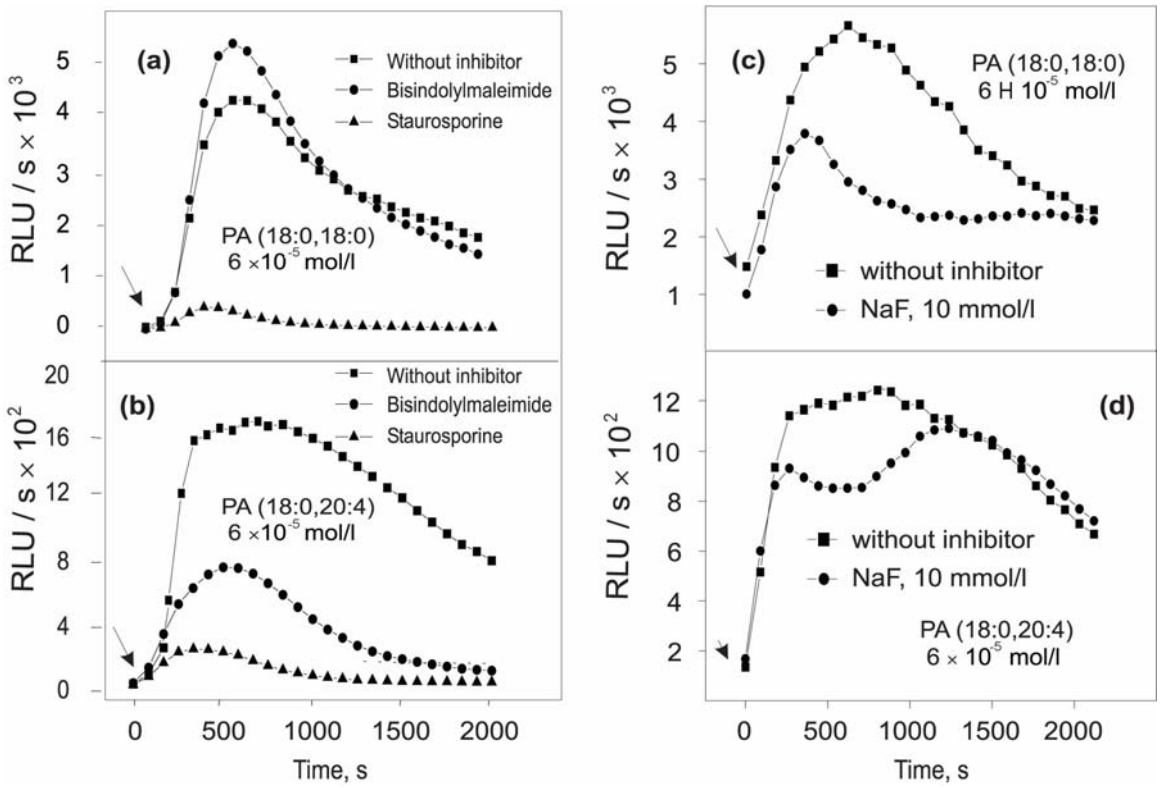

Fig. 2. CL response of PMNs stimulated with DSPA (**a**,**c**) and SAPA (**b**,**d**). An arrow indicates the time of PA addition. In (**a**) and (**b**) cells were preincubated with bisindolylmaleimide (1×10^{-6} mol/l) and staurosporine (0.1×10^{-6} mol/l) and in (**b**) and (**d**) with 1×10^{-2} mol/l NaF prior to the addition of PAs. Luminol was present in all cell suspensions (5×10^{-5} mol/l). CL was monitored for 35 min after the addition of PAs (6×10^{-5} mol/l). The results are typical data of three independent measurements.

An overview of the results obtained from three independent measurements is given in Table 1. None of the peaks of interest was detectable in the organic extract of the unstimulated PMNs. All peaks of PAs are due to the PAs used for stimulation. In the lipid extract of PMNs stimulated with DSPA, the corresponding DAG was not detected, whereas in the lipid extract of PMNs stimulated with SAPA, a small peak corresponding to the sodium adduct of SADG became detectable. No influence of the incubation time of the PMNs with PA was detected (data not shown). Differences between lipid extracts of PMNs stimulated with both PAs and the extract of unstimulated cells were not detected between *m/z* ratios ~ 400 and ~ 580, where lysophospholipids would be expected. Changes in the region between *m/z* ~ 780 and ~ 850 cannot be completely ruled out, since that region is overcrowded with peaks of membranous PLs (Petković *et al.* 2002).

Discussion

In this study we have shown by the luminol-amplified chemiluminescence, that the long-chain (>C14) PAs are able to stimulate ROS production by PMNs, but were ineffective in inducing the degranulation of azurophilic granules in PMNs.

When applied at concentrations lower than 3×10^{-5} mol/l extracellulary added PAs only enhanced the

CL reponse of fMLP-stimulated PMNs (data not shown). At higher concentrations, they actually alone induced the response. CL properties of the PA-stimulated cells were different: DSPA yielded a monophasic curve, whereas SAPA induced a biphasic pattern. Curve shape was comparable to other stimulators known to induce the oxidative activity of PMNs (Fig. 1a), implying some similarities in signaling pathways.

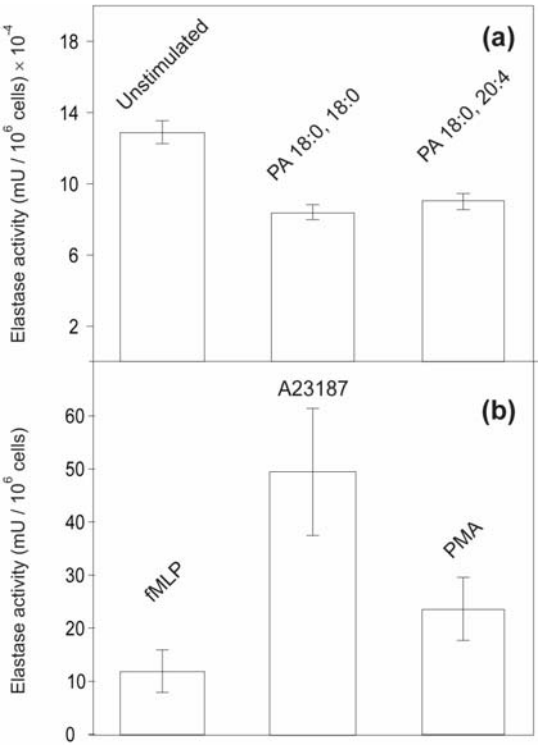

Fig. 3. Elastase activity in the supernatants of PMNs stimulated with DSPA and SAPA at a concentration of 6×10^{-5} mol/l (**a**); In (**b**) the elastase activity in the supernatant of fMLP (10^{-6} mol/l), A23187 (10^{-5} mol/l) and PMA (0.1×10^{-6} mol/l)-stimulated PMNs is shown. Due to large differences in elastase activity scaling in (**a**) and (**b**) is different. PMNs were incubated for 1 min at 37 °C and then the corresponding stimulator was added. The reaction was terminated after 15 min by the addition of ice-cold HBSS, followed by immediate short centrifugation. The obtained supernatant was used for the determination of elastase activity. Elastase activity was measured colorimetrically with *p*-nitroanilide. Results are calculated per cell number and represent the mean value (± S.D.) of three independent measurements.

The question that was addressed in this study was whether the stimulation of the respiratory burst response of PMNs is mediated by PAs as such or whether they have to be first converted into the corresponding DAGs. This conversion could be catalyzed by ecto-PPH even if it was shown that long-chain PAs are rather poor substrates for this enzyme (Perry *et al.* 1993). To check this, MALDI-TOF MS analysis of the lipid extracts of PMNs after stimulation with PAs was performed. The results summarized in Table 1 revealed the presence of a major peak corresponding to SADG in the extract of the cells stimulated with SAPA. This might indicate that SAPA is partially converted into the corresponding DAG. SADG was not detected in the cell extract of the DSPA-stimulated cells. DSDG could not be observed, and this might indicate that – if produced – this species is of very low concentration and below the detection limit of MALDI-TOF device (Petković *et al.* 2001b).

Table 1. Lipid peaks detected in the organic extracts of human PMNs stimulated with PAs. The table comprises the DAGs and PAs detected by positive ion MALDI-TOF MS in organic extracts of human PMNs stimulated with 6×10^{-5} mol/l of PAs. With "-" and "+" the absence or the presence of the corresponding peak, respectively, is indicated. With "++" higher peak intensity is indicated. Abbreviations: MALDI-TOF MS: matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; PMNs human polymorphonuclear leukocytes; DG: diacylglycerol; PA: phosphatidic acid.

	Stimulation Conditions of PMNs		
Peak identity	**Unstimulated**	**DSPA**	**SAPA**
[DSDG + Na]$^+$	–	–	–
[SADG + Na]$^+$	–	–	+
[DSPA + 2 H + Na]$^+$	–	++	–
[DSPA + H + 2 Na]$^+$	–	++	–
[SAPA + 2 H + Na]$^+$	–	–	++
[SAPA + H + 2 Na]$^+$	–	–	++

In addition to these experiments, PMNs were incubated with an inhibitor of ecto-PPH, NaF prior to stimulation with PAs (Taylor *et al.* 1993) and CL response was monitored. These experiments showed that – although a certain level of CL inhibition was observed – the respiratory burst could still be initiated by all PAs (Figs 2c and 2d), even in the presence of higher NaF concentrations (1×10^{-2} mol/l). The inhibitory effect of NaF was, however, more expressed when the cells were stimulated with SAPA. This also implies differences in the signaling pathways triggered by both kinds of PAs: SAPA was most likely first converted into the corresponding DAG that interacts with PKC and stimulates the respiratory burst response, whereas DSPA acts directly. PKC was indicated to be involved in the first phase of the respiratory burst response of PMNs induced by the chemotactic tripeptide, fMLP (Arnhold *et al.* 1999). Similarly, only DAGs with an unsaturated fatty

acid residue, e.g. SADG when added exogenously to the PMN suspension were able to stimulate the respiratory burst response. These results indicate that the signal transduction pathways stimulated by fMLP and SAPA are similar ones. This can also be seen by similar CL properties (Fig. 1).

The CL curve of DSPA-stimulated PMNs was monophasic and it seems that PKC does not play an important role in the signaling pathways triggered by this PA, as bisindolylmaleimide I only slightly inhibited ROS production of DSPA-stimulated cells compared to those stimulated with SAPA (Fig. 2). Furthermore, it seems that DSPA is not converted metabolically into the corresponding DAG, but it might exhibit its effects by interacting with other intracellular enzymes. A potential candidate is PAPK, a staurosporine-sensitive protein kinase involved in the NADPH oxidase activation (Waite *et al.* 1997). This enzyme is postulated to play a role in the latter phases of the respiratory burst response of fMLP-stimulated human PMNs (Arnhold *et al.* 1999).

Although exogenously added PAs stimulated the respiratory burst response of PMNs, they were unable to stimulate the degranulation of azurophilic granules: In comparison to the basal elastase release (from unstimulated cells), the elastase activity in the supernatant of PA-stimulated cells was even lower (Fig. 3a). The ability of cells to release elastase in response to to other stimulators is demonstrated in Figure 3b.

Details of the involvement of lipid-derived second messengers – DAGs or PAs – in the degranulation process of PMNs are still under debate. The results of Korchak *et al.* (1988) and Suchard *et al.* (1994) imply a more important role of DAGs in the fusion of the secretory granules with the plasma membrane. An increase in PA concentrations in the cells did not lead to enhanced degranulation. On the other hand, when intracellular production of PA was decreased by the ethanol addition, the elastase release from PMNs was attenuated (Tamura *et al.* 1998, Vocks *et al.* 2003). The data presented here would emphasize a more important role of DAGs in the degranulation process, but the exact involvement of these two-second messengers in the degranulation processes requires further investigation.

The results of other authors strongly indicate that PMNs possess receptors for short-chain PAs, the specificity of which decreases with increasing chain length of the fatty acid residues. These authors also detected the non-specific distribution of long-chain PAs on the PMN plasma membrane (Siddiqui and English 1996). Our hitherto unpublished observations on the interaction of fluorescently labeled PA with PMNs also support this view. Additionally, it is assumed that the long-chain PAs – resuspended in a detergent-free buffer – ineffectively bind to a putative receptor on the cell surface (English 1996). It is likely that exogenously added long-chain PAs are incorporated into the plasma membrane because all buffers used in this study were prepared without a detergent. This might be an argument against a receptor-mediated effect, although additional experiments must be performed in order to validate that possibility. Therefore, it might be assumed that the effect of those exogenously added PAs could be compared with PAs produced intracellularly.

In summary, we have shown that extracellularly added long-chain PAs can stimulate the respiratory burst response of human PMNs but not the cell degranulation. Although it is most likely that exogenous long-chain PAs interact with the lipids of the PMN membrane, there is still the open question that should be addressed in the near future about the precise localization of PAs or the corresponding DAGs generated from PAs within the cell. Comprehensive experiments are currently conducting in our laboratory to further clarify the role of various lipid second messengers in the degranulation behavior of human PMNs.

Abbreviations

CL: chemiluminescence; DAG: diacylglycerol; DHB 2,5-dihydroxybenzoic acid; 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSDG: 1,2 distearoyl-*sn*-glycerol; DSPA: 1,2-distearoyl-*sn*-glycero-phosphate; fMLP: N-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine; HBSS: Hank's balanced salt solution; LPA: lysophosphatidic acid; MALDI-TOF MS: matrix-assisted laser desorption / ionisation time-of-flight mass spectrometry; NADPH: nicotine amide dinucleotide; PA: phosphatidic acid; PAPK: phosphatidic acid-activated protein kinase; PC: phosphatidylcholine; PI 3-K: phosphatidylinositol 3-kinase; PKC: protein kinase C; PL: phospholipid; PLC: phospholipase C; PLD: phospholipase D; PMA: phorbol 12-myristate 13-acetate; PMN: polymorphonuclear leukocytes; PPH: phosphatidate phosphohydrolase; ROS: reactive oxygen species; SADG: 1-stearoyl-2-arachidonoyl-*sn*-glycerol; SAPA: 1-stearoyl-2-arachidonoyl-*sn*-glycero-phosphate; TFA: trifluoroacetic acid; TRIS: Tris-hydroxymethyl-aminomethane.

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References

ARNHOLD J, BENARD S, KILIAN U, REICHL S, SCHILLER J, ARNOLD K: Modulation of luminol chemiluminescence of fMet-Leu-Phe-stimulated PMNs by affecting dephosphorylation and the metabolism of phosphatidic acid. *Luminescence* **14**: 129-137, 1999.

BENARD S, ARNHOLD J, LEHNERT M, SCHILLER J, ARNOLD K: Experiments towards quantification of saturated and polyunsaturated diacylglycerols by matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry. *Chem Phys Lipids* **100**: 115-125, 1999.

BIETH J, SPIESS B, WERMUTH C: The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochem Med* **11**: 350-357, 1974.

BLIGH EG, DYER WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911-917, 1959.

BØYUM A: Separation of white blood cells. *Nature* **204**: 793-794, 1964.

CASTILLO M, NAKAJIMA K, ZIMMERMANN M, POWERS J: Sensitive substrates for human leukocyte and porcine pancreatic elastase: a study of the merits of various chromophoric and fluorogenic leaving groups in assays for serine proteases. *Anal Biochem* **99**: 53-64, 1979.

ENGLISH D: Phosphatidic acid: a lipid second messenger involved in intracellular and extracellular signalling. *Cell Signal* **8**: 341-347, 1996.

FANTONE JC, WARD PA: Role of oxygen-derived radicals and metabolites in leukocyte-dependent inflammatory reactions. *Am J Pathol* **107**: 397-418, 1982.

GENG D, CHURA J ROBERTS MF: Activation of phospholipase D by phosphatidic acid. Enhanced vesicle binding, phosphatidic acid – Ca^{2+} interaction, or an allosteric effect? *J Biol Chem* **273**: 12195-12202, 1998.

JONES GA, CARPENTER G: Regulation of phospholipase C-gamma 1 by phosphatidic acid. Assessment of kinetic parameters. *J Biol Chem* **268**: 20854-20850, 1993.

KORCHAK HM, VOSSHALL LB, HAINES KA, WILKENFELD C, LINDQUIST KF, WEISSMANN G: Activation of the human neutrophil by calcium-mobilizing ligands. II. Correlation of calcium, diacyl glycerol, and phosphatidic acid generation with superoxide anion generation. *J Biol Chem* **263**: 11098-11105, 1998.

NISHIMURA H, SIMPSON IA: Staurosporine inhibits phorbol 12-myristate 13-acetate- and insulin-stimulated translocation of GLUT1 and GLUT4 glucose transporters in rat adipose cells. *Biochem J* **302**: 271-277, 1994.

NURCOMBE HL, BUCKNALL RC, EDWARDS SW: Activation of the neutrophil myeloperoxidase-H_2O_2 system by synovial fluid isolated from patients with rheumatoid arthritis. *Ann Rheum Dis* **50**: 237-242, 1991.

PERRY DK, STEVENS VL, WIDLANSKI TS, LAMBETH JD: A novel ecto-phosphatidic acid phosphohydrolase activity mediates activation of neutrophil superoxide generation by exogenous phosphatidic acid. *J Biol Chem* **268**: 25302-25310, 1993.

PETKOVIĆ M, SCHILLER J, MÜLLER J, MÜLLER M, ARNOLD K, ARNHOLD J: The signal-to-noise ratio as the measure for the quantification of lysophospholipids by matrix-assisted laser desorption/ionisaton time-of-flight mass spectrometry. *Analyst* **126**: 1041-1050, 2001a.

PETKOVIĆ M, SCHILLER J, MÜLLER M, BENARD S, REICHL S, ARNOLD K, ARNHOLD J: Detection of individual phospholipids in lipid mixtures by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry: phosphatidylcholine prevents the detection of further species. *Anal Biochem* **289**: 202-216, 2001b.

PETKOVIĆ M, SCHILLER J, MÜLLER M, ARNOLD K, ARNHOLD J: Investigation of the lysophospholipid composition of human neutrophils upon different stimulation conditions by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *J Serb Chem Soc* **67**: 149-163, 2002.

PRASAD K, KALRA J, CHANDHARY AK, DEBNATH D: Effect of polymorphonuclear leukocyte-derived oxygen free radicals and hypochlorous acid on cardiac function and some biochemical parameters. *Am Heart J* **119**: 538-550, 1990.

SCHILLER J, ARNHOLD J, SONNTAG K, ARNOLD K: NMR studies on human pathologically changed synovial fluids. Role of hypochlorous acid. *Magn Reson Med* **35**: 848-853, 1996.

SCHILLER J, ARNHOLD J, BENARD S, MÜLLER M, REICHL S, ARNOLD K: Lipid analysis by matrix-assisted laser desorption and ionisation mass spectrometry: a methodological approach. *Anal Biochem* **267**: 45-56, 1999.

SCHILLER J, BENARD S, REICHL S, ARNHOLD J, ARNOLD K: Cartilage degradation by stimulated human PMNs: reactive oxygen species decrease markedly the activity of proteolytic enzymes. *Chem Biol* **7**: 557-568, 2000.

SIDDIQUI RA, ENGLISH D: Phosphatidic acid binding to human PMNs: effect on tyrosine kinase-regulated intracellular Ca^{2+} mobilisation. *Cell Signal* **8**: 349-354, 1996.

SIDDIQUI RA, ENGLISH D: Phosphatidic acid elicits calcium mobilisation and actin polymerisation through a tyrosine kinase-dependent process in human PMNs: a mechanism for induction of chemotaxis. *Biochim Biophys Acta* **1349**: 81-95, 1997.

SIDDIQUI RA, ENGLISH D: Phosphatidylinositol 3'-kinase-mediated calcium mobilisation regulate chemotaxis in phosphatidic acid-stimulated human PMNs. *Biochim Biophys Acta* **1483**: 161-173, 2000.

SIDDIQUI RA, BURTSCHI D, KOVACS R: Phosphatidic acid induces calcium influx in PMNs via verapamil-sensitive calcium channels. *J Cell Biochem* **78**: 297-304, 2000.

SUCHARD SJ, NAKAMURA T, ABE A, BOXER A: Phospholipase D-mediated diradylglycerol formation coincides with H_2O_2 and lactoferrin release in adherent human PMNs. *J Biol Chem* **269**: 8063-8068, 1994.

TAMURA DY, MOORE EE, PATRICK DA, JOHNSON JL, ZALLEN G, SILLIMAN CC: Primed PMNs require phosphatidic acid for maximal receptor-activated elastase release. *J Surg Res* **77**: 71-74, 1998.

TAYLOR GS, LADD A, JAMES J, GREENE B, ENGLISH D: Characterization of phosphatidic acid phosphohydrolase in neutrophil subcellular fractions. *Biochim Biophys Acta* **1175**: 219-224, 1993.

TOULLEC D, PIANETTI P, COSTE H, BELLEVERGUE P, GRAND-PERRET T, AJAKANE M, BOISSIN V, BOURSIER E, LORIOLLE F: The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266**: 15771-15781, 1991.

VOCKS A, PETKOVIĆ M, ARNHOLD J: Involvement of phosphatidic acid in both degranulation and oxidative activity in fMet-Leu-Phe stimulated polymorphonuclear leukocytes. *Cell Physiol Biochem* **13**: 165-172, 2003.

WAITE KA, WALLIN R, QUALLIOTINE-MANN D, MCPHAIL L: Phosphatidic acid–mediated phosphorylation of the NADPH oxidase component p47-*phox*. Evidence that phosphatidic acid may activate a novel protein kinase. *J Biol Chem* **272**: 15569-15578, 1997.

WATSON F: Phospholipases involved in activation of the neutrophil NADPH oxidase. *Methods: A Companion to Methods in Enzymology* **9**: 578-590, 1996.

XIAO H, SIDDIQUI RA, AL-HASANI MH, SLIVA D: Phospholipids released from activated platelets improve platelet aggregation and endothelial cell migration. *Platelets* **12**: 163-170, 2001.

ZHOU D, LUINI W, BERNASCONI S, DIOMEDE L, SALMONA M, MANTO A, SOZZANI S: Phosphatidic acid and lysophosphatidic acid induce haptotactic migration of human monocytes. *J Biol Chem* **270**: 25549-25556, 1995.

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